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The *Arabidopsis thaliana TAG1* mutant has a mutation in a diacylglycerol acyltransferase gene

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Summary

In *Arabidopsis thaliana* (ecotype Columbia) mutant line AS11, an EMS-induced mutation at a locus on chromosome II results in a reduced diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) activity, reduced seed triacylglycerol, an altered seed fatty acid composition, and delayed seed development. A mutation has been identified in AS11 in a gene, which we designated as *TAG1*, that encodes a protein with an amino acid sequence which is similar to a recently reported mammalian DGAT, and, to a lesser extent, to acyl CoA:cholesterol acyltransferases. Molecular analysis revealed that the mutant allele in AS11 has a 147 bp insertion located at the central region of intron 2. At the RNA level, an 81 bp insertion composed entirely of an exon 2 repeat was found in the transcript. While the seed triacylglycerol content is reduced by the lesion in AS11, there is no apparent effect on sterol ester content in the mutant seed. The *TAG1* cDNA was over-expressed in yeast, and its activity as a microsomal DGAT confirmed. Therefore, the *TAG1* locus encodes a diacylglycerol acyltransferase, and the insertion mutation in the *TAG1* gene in mutant AS11 results in its altered lipid phenotype.

Introduction

Plant seed oils are major sources of essential polyunsaturated fatty acids for human diets and renewable feedstocks for chemical industries. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids that are channelled into the cytosolic acyl CoA pool to sustain triacylglycerol accumulation. Triacylglycerol (TAG) biosynthesis is located in the endoplasmic reticulum, with glycerol-3-phosphate and fatty acyl CoAs as the primary substrates. There are three acyltransferases involved in plant storage lipid bioassembly, namely the glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), the *lyso*-phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51) and the diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). These three acyltransferases catalyse the stepwise acylation of the glycerol backbone, with the final step being the acylation of *sn*-1,2-diacylglycerol (DAG) by DGAT into TAGs, a biochemical process generally known as the Kennedy pathway (Stymne and Stobart, 1987). DGAT is the only enzyme in the Kennedy pathway that is exclusively committed to TAG biosynthesis. It has been suggested that DGAT may be one of the rate-limiting steps in plant storage lipid accumulation (Ichihara *et al.*, 1988) and thus a potential target in the genetic modification of plant lipid biosynthesis. However, this hypothesis has not been rigorously tested.

Among the three endoplasmic reticulum-based fatty acyl CoA acyltransferases, only the LPAT gene has been cloned from plants (Knutzon *et al.*, 1995; Lassner *et al.*, 1995). Like several other enzymes involved in storage lipid biosynthesis, acyltransferases are intrinsic ER proteins and are extremely difficult to purify. The research on plant DGAT has been largely limited to studies of activity profiles by using the particulate fractions generated by differential centrifugation of seed- or microspore-derived embryo homogenates (Weselake *et al.*, 1993). Although partial purification of DGAT from cotyledons of germinating soybean seeds was reported (Kwanyuan and Wilson, 1986), detailed molecular characterization of this enzyme is lacking.

We have previously reported the characterization of an EMS-induced *Arabidopsis thaliana* mutant, AS11, with altered fatty acid composition (Katavic *et al.*, 1995). In comparison to wild-type plant seeds, AS11 seeds have reduced levels of the very long chain fatty acid eicosenoic acid (20:1) and reduced oleic acid (18:1) and accumulate α-linolenic acid (18:3) as the major fatty acid in triacylglycerols. The AS11 mutant has a consistently lower ratio of TAG/DAG in developing seeds, and accumulates an elevated amount of seed DAG, the substrate of diacylglycerol acyltransferase. Through a series of biochemical analyses, we showed that AS11 had reduced diacylglycerol acyltransferase activities throughout seed development. AS11 also has a reduced oil content phenotype, providing evidence that DGAT may be controlling flux into TAG biosynthesis. Genetic analysis indicated that the fatty acid phenotype is caused by a semi-dominant mutation in a nuclear gene, designated *TAG1*. The mutation was mapped to chromosome II, and was estimated to lie in the region

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approximately 17.5 ± 3 cM from the *sti* locus and 8 ± 2 cM from the *cp2* locus.

Here we report the identification, functional assignment and cloning of this diacylglycerol acyltransferase-related gene, *TAG1*, from *Arabidopsis thaliana* through a databank sequence search and assisted by map-based information. Our data demonstrate that the encoded product of the *TAG1* gene is homologous to the mammalian DGAT reported recently, and functions as a DGAT.

Results

Isolation of the TAG1 cDNA from Arabidopsis thaliana

Since one of the most likely defects in AS11 mutant is in the DGAT itself, we therefore attempted cloning strategies based on sequence information for enzymes that share common substrates with DGAT. One of the candidate enzymes that would serve this purpose is acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) (Chang *et al.*, 1997). Like DGAT, ACAT is an ER protein functioning as an *O*-acyltransferase by using acyl CoA as the fatty acyl donor for the esterification of free cholesterol to generate sterol esters. Through a BLAST database search, we identified an *Arabidopsis thaliana* expressed sequence tag (EST) (accession number AA042298) with a deduced amino acid sequence showing 41% identity to that of the yeast acyl CoA:cholesterol acyltransferase (Yang *et al.*, 1996; Yu *et al.*, 1996) within the short (104 amino acids) sequence that was available for the EST.

The corresponding cDNA (E6B2T7) clone was obtained from the *Arabidopsis* Biological Resource Center, Columbus, Ohio, USA. Upon complete sequencing, the 878 bp E6B2T7 clone was found to be a partial cDNA. However, the ORF prediction from this partial cDNA confirmed the initial EST search results in that the encoded product is structurally similar to ACAT, especially in the regions at the C-terminus. We were confident that the cDNA contained the 3′ untranslated region through an ORF search, although the polyA tail was missing.

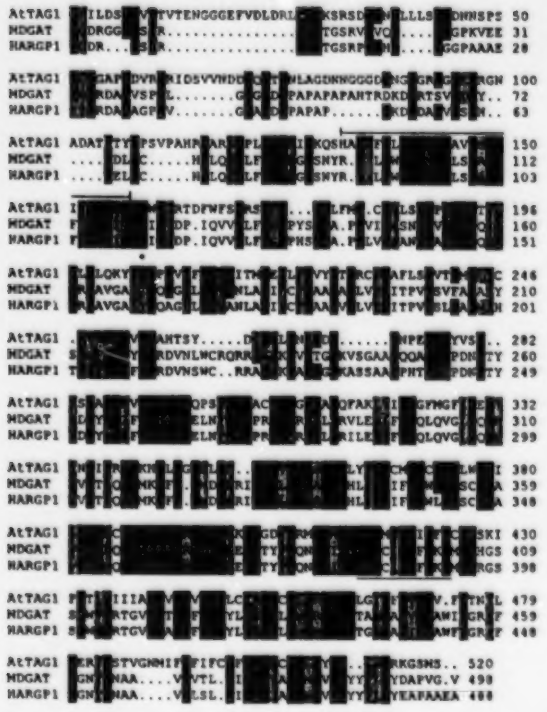
We further used the partial cDNA sequence to search against *Arabidopsis thaliana* genomic sequence information. Subsequently, an *Arabidopsis* 'IGF' BAC clone 'F27F23' (accession number AC003058) was identified to include a region that matches the cDNA, and therefore it was concluded that this is the region encompassing the corresponding gene. Moreover, we were particularly encouraged by the fact that 'F27F23' is contained in the YAC clone, CIC06E08, which, according to the published map position (http://weeds.mgh.harvard.edu/goodman/c2_b.html), represents a region between cM 35.9 and 38.7 on chromosome II, similar to the location estimated for *TAG1* (Katavic *et al.*, 1995). In view of our previous results on the characterization of the AS11 mutant, the map position of this gene strongly suggested that it may encode a gene related to DGAT.

To clone a full-length cDNA, we designed a series of oligonucleotide primers based on the genomic sequences located at different positions 5′ upstream of the region covering the partial cDNA. We used these primers in combination with a primer located at the 3′ UTR of the partial cDNA (E6B2T7) to perform PCR reactions with cDNA phagemid prepared from an *Arabidopsis thaliana* (ecotype Columbia) silique-specific cDNA library (Giraudat *et al.*, 1992) as template. The longest cDNA amplified was 1904 bp, which we subsequently designated as *TAG1*, and deposited into Genbank under accession number AJ238008. We believe this cDNA represents a full-length clone because its size is in agreement with that of the transcript detected in the Northern blot (see below). The longest open reading frame is flanked by a 134 nt 5′ untranslated region and a 203 nt 3′ untranslated region. There is an in-frame stop codon (TGA at position nt 43) which is followed by an in-frame ATG at position nt 139. It is thus inferred that the ATG at position nt 139 is the initiation codon.

The primary structure of TAG1 predicts a DGAT-related enzyme

The predicted open reading frame of the *TAG1* cDNA encodes a polypeptide of 520 amino acids with a calculated molecular weight of 58 993 Da. Using the BLAST search program (Altschul *et al.*, 1990), it was found that the recently reported mouse diacylglycerol acyltransferase (accession number AF078752) (Cases *et al.*, 1998) showed the highest sequence similarity to the deduced amino acid sequence of *TAG1* (Figure 1a). TAG1 was also similar to a human acyl CoA:cholesterol acyltransferase-related enzyme (accession number AF059202). The human acyl CoA:cholesterol acyltransferase-related enzyme, also known as ARGP1, is most likely to be a DGAT with no significant ACAT activity, although the true nature of the enzyme awaits further confirmation (Oelkers *et al.*, 1998). The similarity between TAG1 and the mammalian DGAT extends over a region of more than 400 amino acids with a sequence identity of about 41%. A putative diacylglycerol/phorbol ester-binding motif, HKW-X-X-RH-X-Y-X-P, a signature sequence observed to be unique to DGAT while absent in the ACATs (Oelkers *et al.*, 1998), is located at amino acids 414–424 (Figure 1a). Among other cloned acyltransferases (e.g. GPATs, LPATs, dihydroxyacetone phosphate acyltransferases), it has been reported that there is an invariant proline in a highly hydrophobic block IV that may participate in acyl CoA binding (Lewin *et al.*, 1999). In the TAG1 sequence, the hydrophobic block from residues 221–229 containing an invariant proline at residue 224 might constitute such a motif.

(a)



(b)

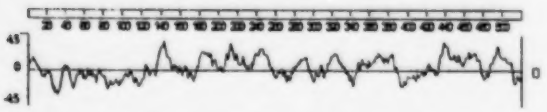


Figure 1. Analysis of TAG1 protein sequence.
(a) Comparison of TAG1 sequence and mammalian DGATs. AtTAG1, *A. thaliana* TAG1; MDGAT, mouse DGAT; HARGP1, human ARGP1 protein. Identical amino acid residues are highlighted in black. Conserved residues are shaded. The overlined segment indicates the insertion generated from the mutation in AS11. The putative diacylglycerol binding site is underlined. The asterisk denotes the SnRK1 potential targeting site.
(b) Kyte–Doolittle hydropathy plot of the TAG1 protein.

TAG1 showed some sequence similarity to other acyl CoA:cholesterol acyltransferases from a number of species (Chang *et al.*, 1997) (data not shown). However, this is largely confined to the *C*-terminus, and is significantly lower (around 30%) than the similarity of TAG1 to the mammalian DGAT.

The TAG1 protein has multiple hydrophobic domains (Figure 1b) and an analysis by the PC Gene program predicted that the protein has five possible transmembrane segments (amino acids 178–195, 233–253, 363–388, 433–476, 486–507). In the mammalian DGAT, a putative tyrosine phosphorylation motif was observed (Case *et al.*, 1998), but no apparent tyrosine phosphorylation site could be found in TAG1. However, a visual examination revealed a consensus sequence (X-L^{200}-X-K^{202}-X-X-S^{205}-X-X-X-V^{209}) identified as a targeting motif typical of members of the SnRK1 protein kinase family. The SnRK1 (SNF1-related protein kinase-1) proteins are a class of Ser/Thr protein kinases that have been increasingly implicated in the global regulation of carbon metabolism in plants (Halford and Hardie, 1998). Interestingly, similar SnRK1 targeting motifs could also be identified in the LPATs from coconut (Knutzon *et al.*, 1995) and meadowfoam (Lassner *et al.*, 1995), respectively.

The TAG1 *gene is ubiquitously expressed in* Arabidopsis

Northern blot analyses were performed to investigate the expression profile of the *TAG1* gene. Total RNA was extracted from different tissues, including roots, leaves, flowers, developing siliques, young seedlings and germinating seeds. We observed the highest steady-state level accumulation of *TAG1* transcripts in RNA isolated from germinating seeds and young seedlings (Figure 2a). *TAG1* transcripts were also detected in root, leaf and flower tissues, albeit at lower levels. Surprisingly, the *TAG1* gene is expressed in developing siliques at a level that is comparable to that of other vegetative tissues, but lower than that of germinating seeds and young seedlings. This expression profile in general is not inconsistent with the notion that DGAT is present in all plant tissues capable of TAG biosynthesis (Kwanyuan and Wilson, 1986). It has been shown in a number of plant species, including soybean and safflower, that germinating seeds actively synthesize TAGs (Ichihara and Noda, 1981; Kwanyuan and Wilson, 1986). The relatively high level of expression in roots is also consistent with the fact that root plastids are capable of synthesizing large amounts of fatty acids to sustain a very active TAG bioassembly process (Sparace *et al.*, 1992).

Southern blot hybridization was performed with genomic DNA digested with several restriction enzymes including *Bgl*II, *Eco*RI and *Hin*dIII. The *TAG1* gene has no internal *Bgl*II and *Hin*dIII sites, while one internal *Eco*RI site exists. Our Southern analysis suggested that *TAG1* most likely represents a single-copy gene in the *Arabidopsis* genome (Figure 2b).

An insertion mutation is found in the TAG1 *gene in mutant AS11*

Alignment of the genomic sequence (accession number AC003058) with that of the *Tag1* cDNA revealed that the

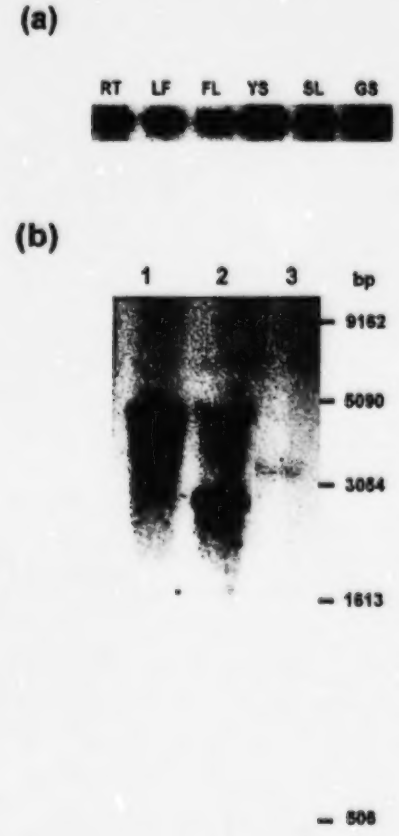


Figure 2. Northern and Southern analyses of the *TAG1* gene.
(a) Northern analysis of *TAG1* gene expression in *Arabidopsis thaliana*. Total RNA was extracted from roots (RT), leaves (LF), flowers (FL), young seedlings (YS), developing siliques (SL) and germinating seeds (GS).
(b) Southern blot analysis of the *TAG1* gene in *Arabidopsis thaliana*. Genomic DNA was digested with restriction enzymes *Bgl*I (lane 1), *Eco*RI (lane 2) and *Hin*dIII (lane 3). The *TAG1* DNA probe was ^{32}P-labelled by random priming.

TAG1 gene contains 16 exons and 15 introns, spanning a region of about 3.4 kb (Figure 3a). DNA containing the *TAG1* allele from AS11 was PCR-amplified and completely sequenced. The AS11 *TAG1* allele has a 147 bp insertion located at the central region of intron 2. The insertion is a duplication of a segment that is composed of 12 bp from the 3′ end of intron 1, the entire sequence of exon 2 (81 bp) and 54 bp from the 5′ end of intron 2.

In order to rule out the possibility of PCR artifacts, two sets of primers were used to perform further PCR amplifications. Primers A and B (see Experimental procedures) located in exons 1 and 3, respectively, amplified a DNA fragment that is about 150 bp longer from AS11 (Figure 3b, lane 2) than that from the wild-type (Figure 3b, lane 1). The second pair of primers, C and B (see Experimental procedures), with one to be found in both exon 2 and the insertion segment, and the other located in exon 3, generated two amplified fragments from AS11 (Figure 3b, lane 4) but only one from the wild-type (Figure 3b, lane 3). Hence these results confirmed that the insertion mutation we identified through sequencing reflected the true nature of the mutation in the *TAG1* gene in the AS11 genome.

The AS11 TAG1 *transcript has an 81 bp insertion in its open reading frame*

Northern blot analyses indicated that there was no difference in the expression profiles of the *TAG1* gene between the AS11 mutant and wild-type *A. thaliana* (data not shown). In order to investigate the effect of the mutation at the transcript level, reverse-transcription PCR (RT–PCR) was performed to amplify the *TAG1* transcript from RNA extracted from germinating seedlings of mutant AS11. Sequencing analysis revealed that there is an 81 bp insertion composed entirely of exon 2 in the transcript from AS11. The exon 2 in the repeat is properly spliced. The alteration of the transcript thus does not disturb the reading frame. However, this additional exon 2 sequence in the AS11 transcript would result in an altered DGAT protein with a 27 amino acid insertion (^{131}SHAGLFNLCVVVLIAVNSRLIIENLMK^{157}). As documented previously, there is a 40–70% reduction in DGAT activity throughout AS11 seed development (Katavic *et al.*, 1995). The 81 bp insert responsible for reduced DGAT activity in AS11 is visible in the comparison of RT–PCR products (compare Figure 3b, lane 5 (WT) and lane 6 (AS11).)

The TAG1 *gene insertion in* Arabidopsis *mutant AS11 affects seed triacylglycerol accumulation, but not sterol ester accumulation in seeds*

Because TAG1 also showed some sequence homology to ACATs from a number of species (Chang *et al.*, 1997), we decided to compare both triacylglycerol and sterol ester accumulation in seeds of the wild-type *A. thaliana* and AS11 mutant. While the triacylglycerol content and TAG/DAG ratios were reduced in AS11, consistent with our previously published findings (Katavic *et al.*, 1995), the proportions of sterol esters in WT and AS11 seeds were similar, at 0.8 and 1% of the total lipid extract, respectively (Table 1). If the *TAG1* lesion affected ACAT-like activity, one might expect a reduction in seed sterol esters, but this was not observed. These results indicated that TAG1 is not involved in sterol ester homeogenesis, and thus is not an acyl CoA:sterol acyltransferase.

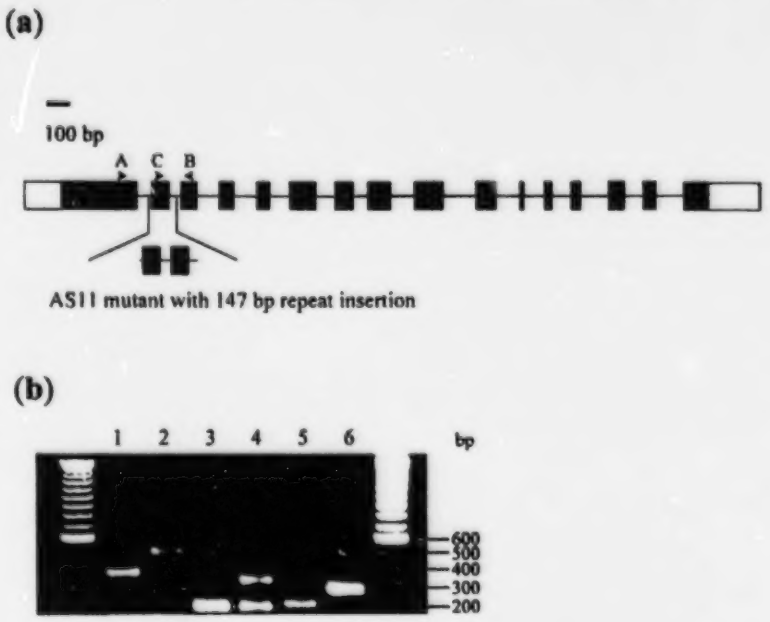


Figure 3. Analysis of the insertion mutation in the *TAG1* gene of AS11.
(a) Diagrammatic representation of the *TAG1* gene structure. The boxes indicate the 15 exons (solid boxes for coding regions, open box for untranslated regions), and the lines represent introns. A, B and C denote the positions of the primers used for PCR amplifications of the segments from wild-type (WT) and AS11.
(b) Gel separation of the PCR products amplified from wild-type (WT) and AS11. Lane 1, PCR product with primers A and B using WT genomic DNA as template. Lane 2, PCR product with primers A and B using AS11 genomic DNA as template. Lane 3, PCR product with primers C and B using WT genomic DNA as template. Lane 4, PCR product with primers C and B using AS11 genomic DNA as template. Lane 5, RT–PCR with primers A and B using RNA prepared from WT seedling RNA. Lane 6, RT–PCR with primers A and B using RNA prepared from AS11 seedling RNA.

Table 1. Comparison of AS11 (Katavic *et al.*, 1995) and wild-type *A. thaliana* seeds with respect to lipid profiles at mid-development, and the relative TAG, DAG and sterol ester contents in AS11 and WT seeds at maturity

Seed type	TAG/DAG ratio		TAG content at maturity		Sterol esters at maturity (% TLE)[d]
	Mid-development[a]	Maturity[a]	Relative[b]	Actual (nmol/mg DW)[c]	
WT	17	90	1.00	255	0.8
AS11	5	20	0.6	174	1.15

[a]Embryos staged and lipids measured as described in Katavic *et al.* (1995).
[b]Relative TAG content of 200-seed samples of AS11 and WT measured by magic angle sample spinning-^{1}H-NMR according to the method of Rutar (1989). The integration response for resonances attributable to liquid-like oil were summed and the value for AS11 seed is reported relative to the response for the WT control seed sample (the latter set at a value of 1.00).
[c]TAG content (nmol/mg dry weight) measured by transmethylation of a TLC-purified TAG fraction, followed by GC analysis of fatty acid methyl esters.
[d]Total lipid extract (TLE) was prepared as described by Taylor *et al.* (1991, 1992) and sterol esters isolated and characterized as described in Experimental procedures.

TAG1 *expression in yeast*

The *TAG1* cDNA over-expressed in yeast resulted in a 3.5–4-fold increase in microsomal DGAT activity compared to plasmid only (*pYES2*) control transformants, strongly suggesting that the *TAG1* gene product functions as a DGAT. This trend was identical in crude lysates (data not shown) and in microsomal fractions prepared by differential centrifugation (Figure 4). In contrast, when ^{14}C18:1-CoA was added to the yeast lysates, sterol esters were also labelled *in vitro* (data not shown), but there was no significant difference in the ^{14}C-labelled sterol esters produced by lysates from the *pYES2* Gal-induced control and the *pYES2:TAG1* Gal-induced transformant. This further suggests that the TAG1 product does *not* encode an acyl CoA:sterol acyltransferase (ACAT homologue).

Discussion

We have identified a gene and cloned its corresponding cDNA, which encodes a protein with significant primary structure similarity to mammalian DGAT. We concluded that this gene is the primary lesion in AS11 based on our previous genetic and biochemical data, physical

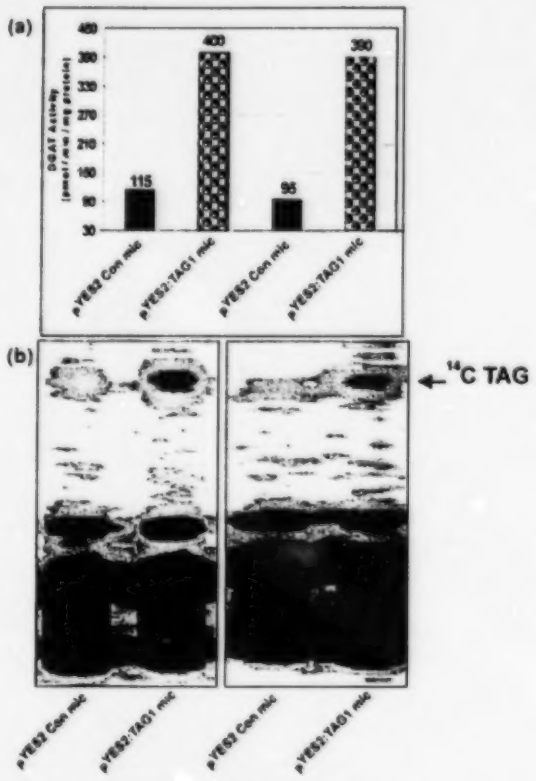


Figure 4. Expression of the *TAG1* cDNA in yeast.
Host cultures of strain YMN5 were transformed with *pYES2* plasmid only (*pYES2* Con; without *TAG1* insert) or with *pYES2* containing the *TAG1* cDNA insert (*pYES2:TAG1*). Following induction in the presence of galactose, transformants were lysed and assayed for DGAT activity as described in Experimental procedures.
(a) The results obtained in two separate DGAT activity experiments performed with microsomal fractions (pYES2Con mic versus pYES2:TAG1 mic) are shown.
(b) Corresponding radio-TLC scans of the microsomal DGAT assay reaction products. The spot intensities are presented on a logarithmic scale. Radiolabelled triacylglycerol bands are indicated by the arrow.

mapping information and gene expression profile, and therefore designated it as *TAG1*. Our previous data indicated that the *TAG1* locus in AS11 has a lesion affecting DGAT activity and consequently the amount and acyl composition of seed triacylglycerols (Katavic *et al.*, 1995). In this study, we have revealed that in AS11, there is an insertion mutation in a gene homologous to the mammalian DGAT that results in an extra exon 2 sequence in its transcript. The DNA aberration observed in this mutant was unexpected, since ethyl methanesuiphonate (EMS) generally causes point mutations. Although we cannot rule out the possibility that this AS11 mutant was the result of a spontaneous mutation event, EMS-induced deletions and insertions have been reported in other systems (Mogami *et al.*, 1986; Okagaki *et al.*, 1991).

The present research allowed us to conclude that the encoded product of the *TAG1* gene functions as a diacylglycerol:acyl CoA acyltransferase. The sequence similarity between TAG1 and the mammalian DGAT as well as the putative diacylglycerol-binding motif favour the probability that TAG1 is a plant DGAT. This was confirmed by over-expression of TAG1 in yeast, and is in accordance with the biochemical defect we observed in AS11.

One could expect that a DGAT gene would be expressed highly in developing seeds, a stage where rapid TAG deposition takes place. Interestingly, the highest level of *TAG1* transcript we could detect was in germinating seeds and young seedlings. It has been shown that DGAT activities are present in various tissues, and the rate of triacylglycerol synthesis in green cotyledons is about half of that in developing seeds (Wilson and Kwanyuan, 1986). In fact, DGAT has been detected and partially purified from germinating soybean cotyledons (Kwanyuan and Wilson, 1986). The AS11 oil content is reduced by about 25–35% (Table 1), with no wrinkled-seed phenotype as described in other low-seed-oil mutants (Focks and Benning, 1998).

The nature of the mutation in *TAG1* would result in a structurally altered DGAT, and our Southern analysis suggested that *TAG1* represents a single-copy gene. Our biochemical data, which measured acyl CoA-dependent DGAT activity, showed that in AS11, there is a reduced DGAT activity (40–70% lower than WT), lower seed TAG and delayed seed development. However, some DGAT activity still remains in AS11, and, while seed development is delayed, significant TAG eventually accumulates. Thus, one possibility is that the lower DGAT activity in AS11 actually results in the delay of seed development. Alternatively, a relatively large proportion of the TAG that is synthesized in AS11 could come from another (possibly seed-specific) DGAT isoform, or from an alternative mechanism, such as diacylglycerol:diacylglycerol transacylase activity (Stobart *et al.*, 1997). It may be that, in the presence of a poorly functioning DGAT, DAG transacylase may play a far more important role than previously thought in catalysing TAG biosynthesis in seeds.

The fatty acyl composition of AS11 seed oil is enriched in 18:3 but greatly reduced in 20:1 and 18:1, and the lower DGAT activity in AS11 results in elevated DAG and reduced TAG levels. In a previous publication (Katavic *et al.*, 1995), we postulated that the reduced DGAT activity in AS11 resulted in repression of very long-chain fatty acid (e.g. 20:1) biosynthesis. However, given our current findings, it is also possible to speculate that, because DAG accumulates and has more time to equilibrate with phosphatidylcholine (via CDP–choline:*sn*-1,2 diacylglycerol choline phosphotransferase, EC 2.7.8.2), this could allow an enrichment in conversion of 18:1 to polyunsaturated C_{18} moieties while esterified to phosphatidylcholine. When combined with the enrichment of the acyl CoA pool with

polyunsaturated C_{18} acyl CoAs released from phosphatidylcholine (via exchange with 18:1-CoA catalysed by the reversible *lyso*-phosphatidylcholine acyltransferase (EC 2.3.1.23)), the end result would be increased 18:2 and 18:3 in both the acyl CoA pool and phosphatidylcholine and DAG pools, with less 18:1-CoA available for elongation. Such a scheme could also explain the acyl composition observed in AS11.

It is unclear why the *TAG1* is expressed highly during seed germination, a stage where lipolytic processes normally take place. It has been suggested that there is subcellular compartmentation of the synthetic and hydrolytic mechanisms associated with the TAG metabolism in germinating cotyledons (Wilson and Kwanyuan, 1986). Although *TAG1* is expressed at a lower level in siliques containing developing seeds, the protein may be very stable. In this context, it should be noted that the mammalian DGAT is expressed at the highest level in the small intestine where fat absorption occurs. In light of the putative protein kinase-targeting motif conserved between TAG1 and LPATs, it is also reasonable to assume that acyltransferase enzyme activities are also regulated at the protein level.

In conclusion, our collective biochemical and cloning and sequencing analysis data from the current and previous studies of mutant AS11, along with the current yeast expression data of the *TAG1* cDNA, suggest that *TAG1* encodes a DGAT isoform ubiquitously present in *A. thaliana*.

Experimental procedures

Plant material

Arabidopsis thaliana ecotype Columbia and mutant AS11 were grown under conditions described previously (Katavic *et al.*, 1995). The *A. thaliana* mutant line AS11 was generated and characterized relative to wild-type (WT) *A. thaliana* ecotype Columbia, as described by Katavic *et al.* (1995).

DNA manipulation

Standard methods and procedures were used for DNA preparation, plasmid propagation and isolation (Sambrook *et al.*, 1989). Sequencing was conducted on an Applied Biosystems Model 373A DNA Sequencing System using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc.). The nucleotide and the deduced amino acid sequences were compared with sequences available in databanks using the BLAST program (Altschul *et al.*, 1990).

Southern and Northern analysis

Total RNA was extracted from different tissues at various developmental stages, using the method of Lindstrom and Vodkin (1991). RNA samples were denatured with formaldehyde and separated on 1.2% formaldehyde–agarose gels. About 5 μg of total RNA was loaded, and the amount of RNA per lane was calibrated by the ethidium bromide-staining intensity of the rRNA bands. Genomic DNA was isolated, digested with restriction enzymes, and a Southern blot analysis was performed according to Sambrook *et al.* (1989). The *TAG1* DNA probe was ^{32}P-labelled by random-priming according to the manufacturer's protocols (BRL).

PCR strategy

Primers used for the amplification of the *TAG1* gene were as follows: DGAT1 (AGACACGAATCCCATTCCCACCGA), DGAT2 (AGTGGTGACAACGCAGGGATGATG), DGAT3 (ATGGTCGCTCCCACATTGTGT), DGAT4 (CATACAATCCCCATGACATTTATCA). DGAT1 and DGAT2 amplify the 5′ half of the *TAG1* gene and DGAT3 and DGAT4 amplify the 3′ end of the *TAG1* gene. Genomic DNA from AS11 was used as template for PCR amplification of the mutant *TAG1* allele using the thermal profile: 94°C for 3 min; 40 cycles of 94°C for 30 sec, 62°C for 45 sec, 72°C for 1 min; and finally 72°C for 15 min. To further confirm the mutation, primers A (CGACCGTCGGTTCCAGCTCATCGG), B (GCGGCCAATCTCGCAGCGATCTTG) and C (TAAACAGTAGACTCATCATCG) were used in pairs (A and B; B and C) to amplify the internal fragment containing the mutation. The primers DGAT1 and DGAT4 were used for PCR amplification of the cDNA with an *A. thaliana* silique cDNA library as template. Primers A and B are also used in RT-PCR amplification of the cDNA fragment encompassing the insertion segment.

Construction of TAG1 *multi-copy vector and transformation and characterization of DGAT expression in yeast*

The *TAG1* cDNA was cloned into pBluescript SK as described (Hadjeb and Berkowitz, 1996). The cDNA was cut out from the vector with *Kpn*I/*Xba*I, and subsquently cloned into the respective sites of the yeast expression vector pYes2 (Invitrogen). The construct was confirmed by sequencing. Constructs with *Tag1* transcription under the control of the *GAL1* promoter released a fragment of approximately 1.9 kb. Because the *TAG1* fragment has its own initiating ATG codon, the product expressed is not a fusion protein. As a host for yeast expression, an *SLC* deletion strain (YMN5 [*slc1*Δ*2*::*LEU2 ura3*]) (kindly provided by M.M. Nagiec and R.C. Dickson, University of Kentucky, Lexington, Kentucky. USA; Nagiec *et al.*, 1993) was used; we reasoned that in this mutant, the endogenous DAG pool may be lower than in WT yeast, and that this would allow us to maximize the activity from over-expressed TAG1 in the presence of exogenously supplied ^{14}C-DAG during *in vitro* DGAT assays of transformant lysates. Yeast transformation was performed according to Elble (1992). YMN5 transformants containing vector only (*pYES2*) were used as controls. Single colonies were cultured overnight in 10 ml of SD medium (synthetic dextrose medium with glucose and without uracil, as described by Ausubel *et al.*, 1995; vol. 2, p. 13.1.3) on a rotary shaker (270 rev/min) at 28°C. The next day, 1 ml of the overnight culture was used to inoculate 99 ml of SD medium. Cells were grown at 28°C for about 17 h. Then 50 ml of this culture were added to 200 ml of SD medium and cells were grown for 4 h. Cells were pelleted, washed once with cold water, and resuspended in 250 ml of medium for induction of expression (SD medium containing 2% galactose (Gal) and without uracil). Cells were reincubated at 28°C, with shaking at 270 rev/min, and

harvested after 4 h. Gal-induced yeast transformants were harvested by centrifugation at 12 500 *g* for 15 min and resuspended in ice cold 100 mM HEPES–NaOH, pH 7.4, containing 1 mM EDTA and 1 mM DTT. All further procedures were carried out at 4°C. Cell lysates were prepared using acid-washed glass beads as described by Ausubel *et al.* (1995). Microsomes were prepared from yeast transformant lysates by differential centrifugation followed by resuspension of the 10 000 *g*–100 000 *g* pellet fraction in grinding medium as described previously (Taylor *et al.*, 1991). Protein in yeast lysates and microsomal fractions were dispersed by sonicating fractions on ice for 2 min in 30 sec cycles with a Labsonic 2000U probe sonicator on the low setting (B. Braun Biotech Inc., Allentown, Pennsylvania, USA). Protein concentrations were measured using the Bradford (1976) assay, protein levels in each fraction were normalized, and equivalent aliquots (250 µg protein) were assayed for DGAT activity as described below.

Lipid substrates and DGAT analyses

^{14}C-labelled diolein [1-^{14}C oleic] (specific activity 55 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA). The ^{14}C-labelled *sn*-1,2-diolein isomer was purified by TLC on borate-impregnated plates and emulsified in HEPES buffer in the presence of 0.2% Tween-20 as described by Taylor *et al.* (1991). 20:1-CoA, CoASH, ATP and all other biochemicals were purchased from Sigma.

DGAT assays were conducted at pH 7.4, with shaking at 100 rev/min in a water bath at 30°C for 30–60 min. Assay mixtures (0.5 ml final volume) contained lysate or microsomal protein (250 µg), 90 mM HEPES–NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1 mM $MgCl_2$, 200 µM *sn*-1,2 diolein [1-^{14}C oleic] (specific activity 2 nCi/nmol) in 0.02% Tween-20, and 18 µM 20:1-CoA as the acyl donor. The ^{14}C-labelled TAGs were isolated by TLC on silica gel G plates developed in hexane:diethyl ether:acetic acid (70:30:1 v/v/v/), the radiolabelled TAG bands visualized on a Bioscan AR-2000 radio-TLC scanner using Win-Scan 2D© software (Bioscan Inc., Washington DC, USA) and the bands scraped and quantified as described by Taylor *et al.* (1991).

Further lipid and sterol ester analyses in AS11 and WT

Total lipid extracts (TLEs) and lipid class analyses in WT and the AS11 mutant were performed as described by Taylor *et al.* (1991, 1992) and Katavic *et al.* (1995). Relative seed oil content was also measured by magic angle sample spinning ^{1}H-NMR, according to the method of Rutar (1989). Analyses were conducted with 200-seed samples of intact wild-type and AS11 seeds using a Bruker AM wide-bore spectrometer (Bruker Analytische Masstechnik GmbH, Karlsruhe, Germany) operating at 360 MHz. To reduce anisotropic line broadening, the seed sample was rotated at 1 kHz in a zirconium rotor oriented 54.7° to the magnetic field. The integration responses for resonances attributable to liquid-like oil were summed and the value for AS11 seed was recorded relative to the response for the WT control seed sample, the latter set at a value of 1.00.

Sterol esters were purified from the TLEs by thin layer chromatography (TLC) on silica H plates developed in hexane:-diethyl ether:formic acid (80:20:2, v/v/v). After elution from the silica H with chloroform:methanol (2:1, v/v), the sterol esters were quantified by saponification followed by methylation of the resulting fatty acids with 3 N methanolic HCl. The fatty acid methyl esters (FAMEs) were analysed by GC as described previously (Taylor *et al.*, 1991). The free sterols released by saponification were also analysed by GC on a 30 m DB-5 column; GC temperature program: initial temperature 180°C, increasing at 10°C min^{-1} to 300°C and held at this temperature for 15 min. The sterol ester content was reported as a percentage of the TLE, i.e. FAMEs released from sterol esters calculated as proportion of the FAMEs released by transmethylation of the total lipid extract.

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GenBank, DDBJ and EMBL mRNA nucleotide sequence database accession number AJ238008 (*TAG1* sequence data).

Note added in proof

Whilst a revision to this manuscript after its review was in progress, another paper describing *A. thaliana* DGAT was published (Hobbs *et al.* (1999) *FEBS Lett.* **452**, 145–149). The two ORFs are identical and Hobbs *et al.* have shown DGAT activity of the ORF product in a recombinant insect cell line culture.